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Drosophila Epsin's role in Notch ligand cells requires three Epsin protein functions: The lipid binding function of the ENTH domain, a single Ubiquitin interaction motif, and a subset of the C-terminal protein binding modules

Xuanhua Xie, Bomsoo Cho, Janice A. Fischer *

Section of Molecular Cell and Developmental Biology, Institute for Cell and Molecular Biology, The University of Texas at Austin, 1 University Station A4800, Austin, Texas 78712, USA

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ABSTRACT

Epsin is an endocytic protein that binds Clathrin, the plasma membrane, Ubiquitin, and also a variety of other endocytic proteins through well-characterized motifs. Although Epsin is a general endocytic factor, genetic analysis in *Drosophila* and mice revealed that Epsin is essential specifically for internalization of ubiquitinated transmembrane ligands of the Notch receptor, a process required for Notch activation. Epsin's mechanism of function is complex and context-dependent. Consequently, how Epsin promotes ligand endocytosis and thus Notch signaling is unclear, as is why Notch signaling is uniquely dependent on Epsin. Here, by generating *Drosophila* lines containing transgenes that express a variety of different Epsin deletion and substitution variants, we tested each of the five protein or lipid interaction modules for a role in Notch activation by each of the two ligands, Serrate and Delta. There are five main results of this work that impact present thinking about the role of Epsin in ligand cells. First, we discovered that deletion or mutation of both UIMs destroyed Epsin's function in Notch signaling and had a greater negative impact on Epsin activity than removal of any other module type. Second, only one of Epsin's two UIMs was essential. Third, the lipid-binding function of the ENTH domain was required only for maximal Epsin activity. Fourth, although the C-terminal Epsin modules that interact with Clathrin, the adapter protein complex AP-2, or endocytic accessory proteins were necessary collectively for Epsin activity, their functions were highly redundant; most unexpected was the finding that Epsin's Clathrin binding motifs were dispensable. Finally, we found that signaling from either ligand, Serrate or Delta, required the same Epsin modules. All of these observations are consistent with a model where Epsin's essential function in ligand cells is to link ubiquitinated Notch ligands to Clathrin-coated vesicles through other Clathrin adapter proteins. We propose that Epsin's specificity for Notch signaling simply reflects its unique ability to interact with the plasma membrane, Ubiquitin, and proteins that bind Clathrin.

Introduction

Epsin is a multi-modular endocytic protein present in metazoans and yeast (Chen et al., 1998; Wendland et al., 1999). Genetic studies in *Drosophila,* nematodes, and mice revealed that Epsin is required specifically for Notch signaling by ligand cells, and probably for all Notch signaling events (Chen et al., 2009; Overstreet et al., 2004; Tian et al., 2004; Wang and Struhl, 2004).

Epsin has a structured N-terminus, called the Epsin-N-terminal homology (ENTH) domain (De Camilli et al., 2001; Kay et al., 1998; Rosenthal et al., 1999) (Fig. 1A). The ENTH domain binds PIP_2 (phosphatidylinositol 4,5-bisphosphate) and also inserts into the plasma membrane and induces membrane curvature (Aguilar et al., 2003; Ford et al., 2002; Itoh et al., 2001). The yeast Epsin (Ent1) ENTH domain also binds Cdc42 GTPase-activating protein (GAP), which may enable Ent1 to coordinate cell polarity with endocytosis (Aguilar et al., 2006).

The unstructured C-terminal region of Epsin contains four different protein–protein interaction motifs in varying numbers in different animal species (De Camilli et al., 2001; Kay et al., 1998) (Fig. 1A). There are two Epsin isoforms in *Drosophila*, produced by alternate splicing of the *liquid facets* (*lqf*) gene pre-mRNA (Fig. 1B) (Cadavid et al., 2000). Each Lqf isoform has two Ubiquitin interaction motifs (UIMs) (Hofmann and Falquet, 2001; Klapsiz et al., 2002; Miller et al., 2004; Oldham et al., 2002; Polo et al., 2002; Shih et al., 2002), two Clathrin binding motifs (CBMs) (Aguilar et al., 2003; Drake et al., 2000), seven DPW motifs that bind the AP-2 endocytic adapter complex (Owen et al., 1999), and two NPF motifs that bind EH-domain-containing endocytic factors such as Eps15 (Aguilar et al., 2003; Paoluzi et al., 1998; Salcini et al., 1997).

The mechanism of Epsin function appears to be complex and context-dependent. Given Epsin's modular structure, the simplest imaginable role for Epsin would be as a Clathrin adapter for transmembrane

* Corresponding author at: The University of Texas at Austin, Moffett Molecular Biology Building, Room 1.312AA, 2500 Speedway, Austin, Texas 78712, USA. Fax: +1 512 232 3432.
E-mail address: jaf@mail.utexas.edu (J.A. Fischer).

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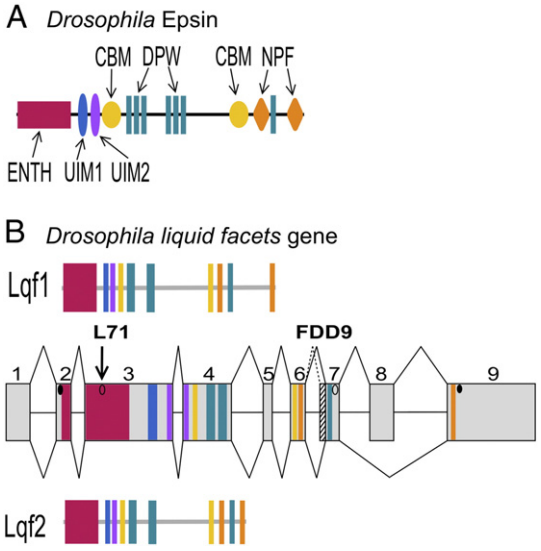

Fig. 1. *Drosophila lqf* gene and Epsin protein structure. (A) A diagram of key features of Epsin protein structure is shown. ENTH = Epsin N-terminal homology domain, UIM = Ubiquitin interaction motif, CBM = Clathrin binding motif, DPW = DPW motifs that bind AP-2, NPF = NPF motifs that bind EH-domain proteins. (B) A diagram of *lqf* genomic DNA is in the center. The nine exons are numbered and the two alternate splice forms of the mRNA are indicated by the bent lines connecting the exons above (*lqf1*) and below (*lqf2*) (Cadavid et al., 2000; Overstreet et al., 2003). The two protein isoforms (Lqf1 and Lqf2) are diagrammed. The colored boxes indicate the different Lqf modules color coded as in (A). The two solid black ovals are the start and stop codons in the wild-type genomic DNA. The unfilled ovals are the stop codons used in the lqf^{L71} and lqf^{FDD9} alleles (Overstreet et al., 2003; see Materials and methods). lqf^{L71} is a nonsense mutation (Overstreet et al., 2003; Wang and Struhl, 2004). lqf^{FDD9} is a temperature sensitive allele with a point mutation (G-to-A) that generates a cryptic splice acceptor site in intron 6 resulting in production of a single Epsin protein that is C-terminally truncated (the final 11 nucleotides of intron 6 become the 5′-end of exon 7, resulting in a 10 aa insertion and an early stop codon) and present at levels much lower than wild-type Epsin (Overstreet et al., 2003 and Materials and methods). The dotted line indicates the 3′-splice acceptor site used by the lqf^{FDD9} allele, and the lined box shows the addition to exon 7 present in lqf^{FDD9} mRNA. lqf^{ARI} (not shown in diagram) is a small deletion 3′ to the transcribed region (Cadavid et al., 2000). lqf^{ARI} produces no detectable protein (Overstreet et al., 2003).

cargo that use Ubiquitin as an endocytosis signal (Aguilar et al., 2003). Epsin might link transmembrane cargo with Clathrin cages either directly, or indirectly through AP-2 (Aguilar and Wendland, 2006b). However, there is evidence that the function of Epsin may be much more complex than that. For one, while the results of some studies suggest that Epsin functions in Clathrin-dependent endocytosis (Chen et al., 1998; Hawryluk et al., 2006), other results suggest that Epsin functions also in Clathrin-independent membrane internalization (Sigismund et al., 2005). In addition, the ENTH domain of yeast Epsin (Ent1) has a function separate from its role in endocytosis. The Ent1 ENTH domain alone is sufficient for the essential function of the protein in yeast, which is not endocytosis, but the regulation of actin cytoskeleton dynamics (Aguilar et al., 2006). Finally, opposing roles for Epsin's UIMs and CBMs have been proposed. The UIMs are required for Epsin ubiquitination (Hofmann and Falquet, 2001; Klapsiz et al., 2002; Miller et al., 2004; Oldham et al., 2002; Polo et al., 2002; Shih et al., 2002) and Epsin ubiquitination inhibits Epsin function (Chen et al., 2002). Also, in vertebrate cell culture it has been reported that Clathrin binding through the CBMs antagonizes UIM binding to Ubiquitin (Chen and De Camilli, 2005).

Epsin is required in Notch ligand (signaling) cells for receptor activation in adjacent signal receiving cells (Overstreet et al., 2004; Tian et al., 2004; Wang and Struhl, 2004). The Notch receptor and its ligands (Delta and Serrate, in *Drosophila*) are transmembrane proteins. Receptor activation leads ultimately to cleavage of an intracellular receptor fragment that enters the nucleus and acts as a transcription factor (reviewed in Bray, 2006). There is compelling evidence that ligand cells must internalize ligand in order to signal (reviewed in: Le Borgne et al., 2005; Nichols et al., 2007a, 2007b; Weinmaster and Fischer, 2011). There is also persuasive evidence that Epsin's function in ligand cells is to promote Clathrin-dependent ligand endocytosis. For one, *lqf* interacts genetically with a variety of genes that encode endocytosis proteins, including Clathrin (Banks et al., 2011; Cadavid et al., 2000; Eun et al., 2007, 2008; Hagedorn et al., 2006). Second, Notch ligands sometimes accumulate abnormally at the plasma membrane in the absence of Epsin (Eun et al., 2008; Overstreet et al., 2004; Wang and Struhl, 2004, 2005). Finally, ubiquitination of Notch ligands is required for signaling (reviewed in Weinmaster and Fischer, 2011) and promotes ligand endocytosis that depends on Epsin (Wang and Struhl, 2004). As genetic evidence indicates a positive role for Clathrin in Notch signaling and Epsin has both UIMs and CBMs, Epsin is implicated as the link between ligands and Clathrin-coated vesicles. Yet, this simple model has not been tested experimentally.

Notch signaling is unique in its requirement for both ligand endocytosis and Epsin, suggesting that Epsin might play a particular role in a special form of endocytosis. Ligand internalization by the signaling cells is most likely required to exert a pulling force on Notch that activates the receptor (reviewed in: Le Borgne et al., 2005; Nichols et al., 2007a, 2007b; Weinmaster and Fischer, 2011). It has been speculated that generation of the pulling force may require a special endocytic structure whose formation depends on Epsin's ability to organize actin at the plasma membrane (see Weinmaster and Fischer, 2011). Ligand transcytosis (recycling) is also required for Notch signaling in some cell types (reviewed in Weinmaster and Fischer, 2011). In this process, Epsin may define a special Clathrin-mediated endocytic pathway, distinct from the typical AP-2-dependent pathway, that routes ligand into distinct endosomes (see Wang and Struhl, 2004). Remarkably, either the ENTH domain of *Drosophila* Epsin, which has both endocytic and actin organizing functions, or the remaining C-terminal endocytic modules, provides significant Epsin activity when overexpressed (Overstreet et al., 2003). Thus, it is unclear which function of Epsin – the actin regulatory or endocytic function of the ENTH domain, or the endocytic function of C-terminus – or all of them – are important for Notch signaling under physiological conditions.

The specific requirement for Epsin in Notch ligand cells means that Epsin is somehow intrinsic to the mechanism by which ligand cells activate Notch receptors in adjacent cells. An important step forward in understanding the role of Epsin in Notch ligand cells is to determine which Epsin modules are required for this process in particular. Here, by generating a variety of transgenes that express amino acid deletion or substitution variants of Epsin in transformed flies, we tested each Epsin module type to determine whether or not it was needed for Notch signaling. The results suggest that in its role in Notch ligand cells, Epsin is divided into three necessary functional regions: the lipid binding function of the ENTH domain, a single UIM, and the C-terminus. The results are most consistent with a model where Epsin recognizes ubiquitinated ligands at the plasma membrane and links ligands to Clathrin indirectly through interactions with other endocytic proteins, including the conventional AP-2 adapter. We propose that Epsin's apparent specificity for Notch signaling is not because Epsin generates a special endocytic environment, but because Epsin is unique in its ability to interact with the plasma membrane, ubiquitinated transmembrane cargo, and a variety of other proteins present in Clathrin-coated vesicles.

Materials and methods

Drosophila strains

The following mutant alleles of *lqf*, maintained in our laboratory, were used: lqf^{FDD9} (FBal0104483), lqf^{ARI} (FBal0104485), lqf^{L71} (FBal0147029). Chromosomes used: *hs-flp tub-gal4 UAS-ngfp* on X

chr. (G. Struhl); *mδ0.5-lacZ* on chr. 2 (S. Bray); *FRT80B lqf*^{L71} (G. Struhl); *tub-gal80 FRT80B*, *FRT82B tub-gal80* (Bloomington); *FRT82B Ser*^{RX106} (Y.N. Jan); *FRT82B Dl*^{rev} (N. Baker); *FRT82B Dl*^{rev} *Ser*^{RX82} (N. Baker).

Molecular biology

Enzymes were from Promega Biotech, New England BioLabs, and Boehringer Mannheim. Herculase polymerase (Stratagene) was used for PCR. DNA sequences of all PCR amplification products were verified. Automated fluorometric DNA sequencing was performed in the DNA analysis facility of the Institute for Cell and Molecular Biology (ICMB) at UT Austin.

Generation of Epsin deletion P element and PhiC31 integrase transgenes and transformants

Deletion mutants (except for *ΔUIM* and *ΔUIM2*) were generated by a PCR-based method (Fig. S1). This PCR method was also used to fuse GFP in-frame to the Epsin C-terminus (Fig. S1). A list of primers used for each construct, complete construction details, and the amino acid content of each Epsin variant are in Supplemental Text. All constructs were ligated ultimately as *Not I–Xho I* fragments into *pCaSpeR4* (Thummel and Pirrotta, 1992) or into an *attB* vector that we constructed called *pCaSper4-attB*. Complete details of the vector construction and a map are in Supplemental Text. P element transformation was by Genetic Services (Sudbury, MA) or Genetivision (Houston, TX) and PhiC31 integrase transformation (into site VK37 at polytene band 22A3) was by Genetivision.

Analysis of eyes and wings

Adult external eyes were photographed with an Olympus SZX12 microscope equipped with a SPOT idea (Diagnostic Instruments) camera. Plastic sectioning of adult eyes was as described (Tomlinson and Ready, 1987). Eye sections were photographed with a Zeiss Axioplan equipped with an Axiocam Hrc. For immunostaining, eye discs in Figs. S2 and S3 were fixed in PEMS and antibody incubations and washes were in PBST (Fischer-Vize et al., 1992). The antibodies used in Figs. S2 and S3 were: (A, B) guinea pig anti-Lqf (1:1000) (Chen et al., 2002) and 488-donkey anti-guinea pig (1:200) (Jackson Laboratories), rat anti-Chc (1:100) (Wingen et al., 2009) and 647-goat anti-rat (1:200; Molecular Probes); (C, D) chicken anti-GFP (1:1000) (AbCam) and 488-goat anti-chicken (1:800) (Jackson Laboratories); anti-Chc as in (A, B). The eye discs in Fig. 3 and all wing discs were immunostained as follows. Discs were fixed in PEMS buffer with 1.0% NP-40 for 15 min. Antibody treatment was as described previously (Lim et al., 2007) with modifications. Fixed discs were blocked for 2 h. at 4 °C in 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 0.5% NP-40 and 5 mg/ml BSA, and then incubated in primary antibody diluted in blocking solution overnight at 4 °C. Discs were washed in 50 mM Tris–HCl (pH 7.4), 150 mM NaCl and 0.5% NP-40 3× for 5 min., and incubated with secondary antibodies in washing solution for 2 h at room temperature, and then washed 3× for 5 min. Primary antibodies were: mouse monoclonal anti-Cut at 1:100 from (Developmental Studies Hybridoma Bank (DSHB)), mouse monoclonal anti-β-galactosidase at 1:50 from the DSHB, rabbit anti-Svp at 1:100 (Ryan et al., 2005). Secondary antibodies (1:200; Molecular Probes) were: Alexa^{568}-anti-rabbit, Alexa^{568}-anti-mouse, Alexa^{568}-anti-guinea pig, Alexa^{633}-anti-mouse, Cy5-anti-rabbit. Phalloidin treatment of eye and wing discs (568-phalloidin, Invitrogen) was as described (Chen et al., 2002). Eye and wing discs were mounted in Vectashield (Vector) and photographed with a Leica TCSSP2 or SP2AOBS confocal microscope. Images were processed with Adobe Photoshop CS3. MARCM clones (Lee and Luo, 1999) were generated by heat shocking first or second instar larvae at 37 °C for 60 min.

Quantitative Western analysis

Epsin-GFP variants expressed by transgenes were quantified using Western blots using anti-GFP and anti-β-tubulin, and compared to endogenous Epsin indirectly through one copy of *FL*. Eye disc protein extracts were generated and analyzed on Western blots as described (Chen et al., 2002), probed with guinea pig-anti-Lqf (1:1000) or mouse-anti-GFP (1:1000) from Santa Cruz Biotechnology, and mouse mAbE7 (anti-β-tubulin from DSHB) at 1:100. Secondary antibodies were HRP-anti-guinea pig (Jackson) at 1:20,000, HRP-goat-anti-mouse (Sigma) at 1:2000 and HRP-anti-mouse (Santa Cruz Biotechnology) at 1:500. The results were quantified using NIH Image J.

Analysis of lqf^{FDD9} *mRNAs*

RT-PCR (Invitrogen) of total mRNA from five *lqf*^{FDD9} third instar larvae (Qiagen RNAeasy Mini Kit) amplified only the mutant splice form. Thus we infer that only mutant protein is produced from the *lqf*^{FDD9} allele. The primers were 5′-ATTCCAGCAACAGCAGCCAG-3′ (in exon 6) and 5′-TGCTGACTGAAAACGGGGC-3′ (in exon 7). These primers amplified a 146 bp fragment of *lqf* genomic DNA, a 71 bp fragment of wild-type *lqf* mRNA, and an 82 bp fragment of *lqf*^{FDD9} mRNA. Both the 71 bp and 82 bp bands were detected in mRNA from *lqf*^{FDD9}/*lqf*+ heterozygotes.

Protein interaction experiments

The Gateway (Invitrogen) vectors *pDEST15* (GST fusions) or *pVP13* (MBP fusions) (Thao et al., 2004) were used to express proteins in bacteria according to manufacturer's instructions (Invitrogen). Details of the plasmid constructions are in Supplemental Text. The fusion proteins were purified from BL21 Rosetta Cells (Novagen) according to the procedure at http://wolfson.huji.ac.il/purification/TagProteinPurif/MBP_Tag_nature.html. Proteins were quantified on Coomassie stained gels with Precision Plus Protein Unstained Standards (Bio-Rad). GST-tagged proteins were immobilized on glutathione Sepharose 4B (GE Healthcare) by mixing 1 ml of bacterial cell lysate with 200 ul Sepharose prewashed with GST binding buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 10% glycerol, 0.5% NP-40; just before use, 10 μl 1 M DTT and 1 tablet Protease Inhibitor Cocktail (Roche) added per 10 ml buffer). A 30 μl aliquot of the GST-fusion loaded Sepharose was used for protein quantification as described earlier. The GST pull-down procedure used is modified from Drake et al., 2000. Aliquots (30 μl) of loaded Sepharose were mixed with MBP-Epsin proteins (the same weight as GST-fusion protein) and assay buffer (25 mM Hepes-KOH ph 7.2, 125 mM potassium acetate, 2.5 mM magnesium acetate, 5 mM EGTA, 1 mM DTT) added to make the total volume 100 μl. After overnight incubation at 4 °C with continuous gentle mixing, the Sepharose beads were recovered by centrifugation at 10,000 *g* for 1 min. The supernatant was removed and a portion of that (the "S" fraction) was mixed with SDS-PAGE buffer. The Sepharose pellets were washed 5× with 1 ml ice-cold PBS and after centrifugation, the final supernatants were aspirated and a portion of each pellet (the "P" fraction) was resuspended in SDS-PAGE buffer. GST-Ub experiment: GST-Ub was 0.6 μg/μl and MBP-Epsins were 0.2 μg/μl. The S lane on the gel was 10 μl of a dilution (10 μl supernatant adjusted to 80 μl with SDS-PAGE buffer) and the P lane was 10 μl of the pellet resuspended in 30 μl. GST-ChcTD experiment: GST-ChcTD was 0.1 μg/μl and MBP-Epsins were 1 μg/μl. The S lane on the gel was 20 μl of a dilution (25 μl supernatant adjusted to 120 μl) and the P lane was 20 μl of the pellet resuspended in 50 μl. GST-α-AdaEar experiment: GST-α-AdaEar was 0.2 μg/μl and MBP-Epsins were 0.2 μg/μl. The S lane on the gel was 20 μl of a dilution (5 μl supernatant adjusted to 80 μl) and the P lane was 20 μl of the pellet resuspended in 80 μl. GST-Eps15EH experiment: GST-Eps15EH was 0.4 μg/μl and MBP-Epsins were 0.4 μg/μl. The S lane on the gel was

20 μl of a dilution (25 μl supernatant adjusted to 120 μl) and the P lane was 20 μl of the pellet resuspended in 50 μl.

Results

Deletion of individual Epsin module types: the UIMs are the single module type most important to Epsin's ability to promote ligand signaling

The first aim was to generate *Drosophila* lines that express, at levels as close to the endogenous Epsin (*lqf*) gene as possible, one of five different variants of Epsin in which all copies (or most copies in the case of the DPWs) of a particular module type were deleted. To this end, we first generated a 16 kilobase *Drosophila* genomic DNA fragment containing a *lqf* gene tagged at the 3′ end with *gfp*, and transformed flies with it using a P element vector. (The GFP tag is mainly to facilitate quantitation of protein expression levels.) A single copy of this transgene, called *FL* for "full length", complemented completely the embryonic lethality of *lqf* null mutants (lqf^{L71}/lqf^{ARI}) and the rescued adults were normal morphologically (Fig. 2). Moreover, endogenous Epsin and Epsin-GFP from the *FL* transgene each colocalized extensively with Clathrin in eye discs and were present mainly in puncta at the apical plasma membrane (Fig. S2). Next we generated the five deletion derivatives of *FL* shown in Fig. 2A. The Epsin-GFP proteins expressed by each transgene in several different transformant lines were quantified using Western blots of eye disc protein extracts (Materials and methods) and lines that matched normal endogenous Epsin levels most closely were used for further analysis (Fig. 2A).

Rescue of lethality in lqf nulls and eye defects in lqf hypomorphs

First, we tested whether or not a single copy of each transgene could substitute for the endogenous gene as well as *FL* did. We found that three of the transgenes – *ΔCBM,ΔDPW*, and *ΔNPF* – did so; each complemented *lqf* null mutants completely (Fig. 2). (In the ΔDPW construct, the two clusters of 3 DPW motifs are deleted leaving a single motif which would not be expected to bind AP-2 significantly (Owen et al., 1999)). Although *ΔENTH* did not rescue the *lqf* nulls completely, it did retain significant Epsin activity as some viable adults eclosed with morphological defects typical of Notch signaling mutants (including malformed eyes, wings, and legs) (Fig. 2). In contrast, *ΔUIM* had almost no lqf^{+} activity; the developing animals died as pupae and no escapers ever eclosed (Fig. 2). To better detect and resolve the low levels of lqf^{+} gene activity in the *ΔENTH* and *ΔUIM* transgenes, we also tested how well each of the transgenes rescued the morphological defects of *lqf* hypomorphs (lqf^{FDD9} homozygotes, Fig. 2A). lqf^{FDD9} homozygotes are semi-viable with typical Notch pathway gene mutant defects, including abnormal eye morphology (Cadavid et al., 2000). Consistent with the idea that *ΔENTH* retained significant lqf^{+} gene activity and *ΔUIM* did not, *ΔENTH* rescued lqf^{FDD9} to wild-type while *ΔUIM* rescued the lqf^{FDD9} defects only slightly (Fig. 2A). The absence of UIMs or CBMs did not prevent ΔUIM nor ΔCBM from accumulating at the plasma membrane similarly to wild-type Epsin-GFP (FL) (Figs. S2, S3). ΔENTH protein also accumulates at the plasma membrane, but to a lesser extent than FL (Figs. S2, S3). Therefore, deletion of the ENTH domain may lower Epsin activity at least in part by preventing efficient localization of Epsin to the plasma membrane (see later discussion).

Rescue of Delta-mediated Notch activation in the eye

In order to test directly whether expression of ΔUIM in ligand cells affects Notch activation in adjacent cells, we used the MARCM technique (Lee and Luo, 1999) to generate GFP-marked lqf^{-} (*lqf* null) cell clones in eye discs that either do or do not contain an Epsin transgene — either *FL* or *ΔUIM*. (GFP signal from the transgenes was invisible in these and subsequent imaginal disc clone experiments because Epsin-GFP was expressed

A

construct	protein level	rescue of *lqf*- null	rescue of *lqf*- hypomorph
none	1.0	embryonic lethal	viable; Notch-like defects (10±1% wild-type facets)
FL	0.4-1.2	wild-type	wild-type
ΔENTH	0.8	semi-viable; Notch-like defects	wild-type
ΔUIM	2.4	pupal lethal	viable; rescued partially (70±4% wild-type facets)
ΔCBM	0.5	wild-type	wild-type
ΔDPW	0.9	wild-type	wild-type
ΔNPF	1.4	wild-type	wild-type

B rescue of *lqf* null

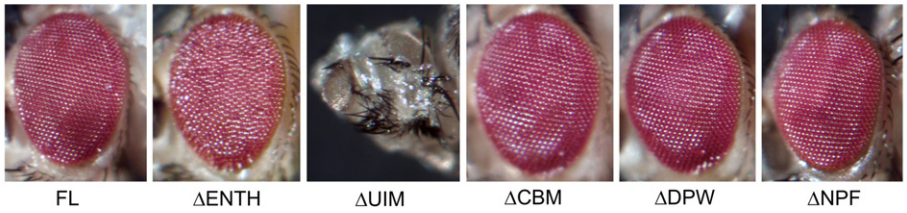

Fig. 2. Epsin's ENTH domain and UIMs are each required for Notch signaling. (A) At left are diagrams of full-length (FL) Epsin-GFP protein and five deletion derivatives (described in text) expressed by P element transgenes. Protein levels from a single transgene copy were measured in eye discs and normalized to the amount of protein produced by one endogenous *lqf* gene copy as described in Materials and methods. How well a single copy of each transgene rescues the mutant phenotypes of *lqf* nulls (lqf^{L71}/lqf^{ARI}) and *lqf* hypomorphs (lqf^{FDD9}) is indicated. (The *lqf* mutant alleles are described in Fig. 1.) The fraction of wild-type facets was obtained by counting ~100 facets in each of at least 3 adult eyes. Wild-type eyes have 100% wild-type facets. (B) External eyes of flies (or pupae for *ΔUIM*) that are lqf^{L71}/lqf^{ARI} and have a single copy of the transgene indicated. All of the eyes are like wild-type except for *ΔENTH* (the external eyes are slightly roughened due to defects in retinal development) and *ΔUIM* (dying pupae dissected from the pupal case have no eyes.).

at much lower levels than the nuclear GFP that marks the clones.) In the eye discs, we monitored Notch activation in the R3/R4 photoreceptor cell pair using a transgene called *mδ0.5-lacZ* that is transcribed in response to Notch activation (Cooper and Bray, 1999). Normally, Delta signaling by the equatorial cell (R3) activates Notch in the polar cell (R4) and thus R4 expresses *mδ0.5-lacZ* (Fig. 3A–A″) (Cooper and Bray, 1999; Fanto and Mlodzik, 1999; Tomlinson and Struhl, 1999). In mosaic R3/R4 pairs in which R3 is *lqf*$^{-}$ and R4 is *lqf*$^{+}$, R3's ability to signal through Delta was impaired and R4 did not express *mδ0.5-lacZ* (0/36 pairs restored; Fig. 3B). In contrast, *FL* largely restored the ability of R3s to activate Notch in adjacent R4s (25/31 pairs restored; Fig. 3C,C′), while *ΔUIM* expression did not (2/47 pairs restored; Fig. 3D,D′). We conclude that in Delta signaling cells, Epsin requires its UIMs to activate Notch in adjacent cells.

Rescue of Delta- or Serrate-mediated Notch activation in the wing

Both Delta and Serrate require Epsin to signal (Wang and Struhl, 2005). We used wing discs to ask if like Delta, the ligand Serrate requires Epsin's UIMs. Notch is activated in a stripe of cells at the dorsal/ventral (D/V) boundary of the wing disc by Delta from the ventral side and Serrate from the dorsal side (Fig. 4A) (reviewed by Irvine and Vogt, 1997; Irvine, 1999 and Blair, 2000). In response to Notch activation, the transcription factor Cut is expressed at the D/V boundary. In clones of *Dl*$^{-}$ *Ser*$^{-}$ cells (Fig. 4B,B′) or *lqf*$^{-}$ cells (Fig. 4C,C′) (Wang and Struhl, 2004) that span the D/V boundary, Cut expression is absent. Expression of *FL* restores Cut expression in *lqf*$^{-}$ cells (Fig. 4D,D′), but *ΔUIM* expression does not (Fig. 4G,G′). We conclude that Epsin's UIMs are required for signaling by Serrate as well as by Delta.

We also used wing discs to ask if Delta and Serrate have the same requirements for other Epsin modules. In *lqf*$^{-}$ clones that span the D/V boundary we saw Cut expression restored to apparently normal levels by *ΔENTH*,*ΔCBM*, *ΔDPW*, or *ΔNPF* (Fig. 4E–F′ and data not shown). As this result may be observed sometimes in clones that are only *Dl*$^{-}$ or only *Ser*$^{-}$ (Micchelli et al., 1997), to determine if in the clones both Delta and Serrate were signaling, we analyzed dorsal and ventral clones that abut the D/V border. Clones generated after the first larval instar stage do not cross the D/V midline (Garcia-Bellido et al., 1976). Ventral *Dl*$^{-}$ clones that abut the D/V border can receive but not send signals and thus Cut is expressed inside the clone but not in adjacent midline cells (Fig. 5A; Glittenberg et al., 2006). In contrast, dorsal *Dl*$^{-}$ clones can send and receive signals (Fig. 5A; Glittenberg et al., 2006). Conversely, dorsal *Ser*$^{-}$ clones can receive but not send, while ventral *Ser*$^{-}$ clones can both send and receive (Figs. 5A, S4). We found that each of the four transgenes (*ΔENTH*,*ΔCBM*, *ΔDPW*, or *ΔNPF*) restored the ability of *lqf*$^{-}$ cells to signal from either the dorsal or ventral side (Fig. 5B–G and data not shown). We conclude that in the context of an otherwise intact Epsin protein, neither Delta nor Serrate requires the ENTH domain, the CBMs, the DPWs, nor the NPF motifs of Epsin to signal.

Differences in the assays

The embryonic lethality of *lqf* null mutants as well as the defects later in development are probably due entirely to the failure of Notch signaling, and thus the transgene complementation test is a reasonable assay for Epsin activity in the Notch pathway. This assumption is based on several observations. First, analysis of *lqf* null clones in developing

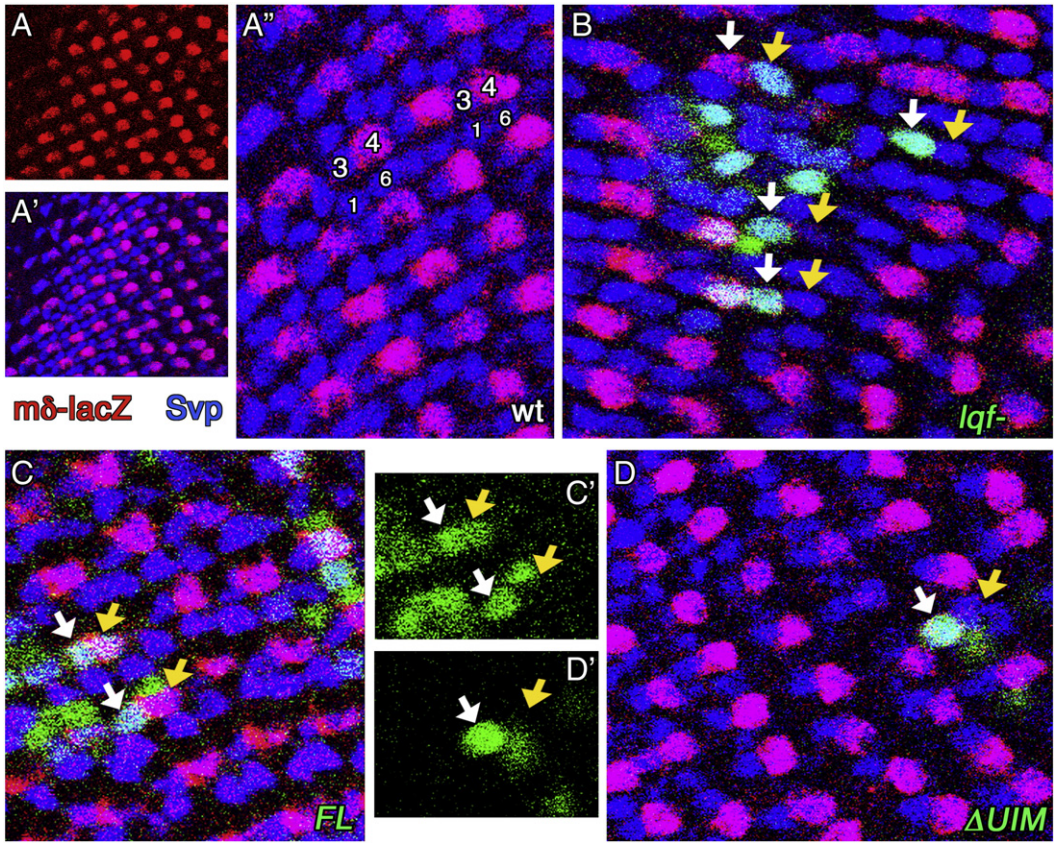

Fig. 3. Ligand cells in the eye disc require Epsin's UIMs to signal. Confocal microscope images of third instar larval eye discs are shown. (A–A″) In otherwise wild-type (wt) discs containing the Notch activation reporter transgene *mδ0.5-lacZ* , Notch is activated in R4 (red nuclei express β−galactosidase). Seven-up (Svp) protein marks the nuclei of R3/R4 and R1/R6 (blue; Mlodzik et al., 1990), distinguishable by their invariable positions (3,4,1,6 in A″). A″ in an enlargement of the center of A′. The morphogenetic furrow is left. (B-D) The green cells are *lqf* null, and the blue and red are as in A″. (B) A MARCM clone in a disc of the genotype *hs-flp tub-gal4 UAS-ngfp*/+; *mδ0.5-lacZ*/+; *lqf*^{k71} *FRT80B*/*tub-gal80 FRT80B* is shown. The green cells are *lqf* null, and the blue and red are as in A". Arrows indicate mosaic pre-R3/pre-R4 pairs where a *lqf*− pre-R3 (white arrow) is adjacent to a *lqf*+ pre-R4 (yellow arrow) in which Notch is not activated (the nucleus is not red). (C) A MARCM clone in a disc of the same genotype as in B, except it also has a second chromosome containing the *FL* transgene. Arrows indicate mosaic pre-R3/pre-R4 pairs where a *lqf*$^{-}$ pre-R3 (white arrow) is adjacent to a pre-R4 (yellow arrow) that is either *lqf*+ or *lqf*$^{-}$ and Notch is activated (the nucleus is red) in both cases. (C′) An enlargement of the clone in C showing only the GFP signal to clarify which cells are *lqf*$^{-}$ (green) and which are *lqf*$^{+}$ (not green).

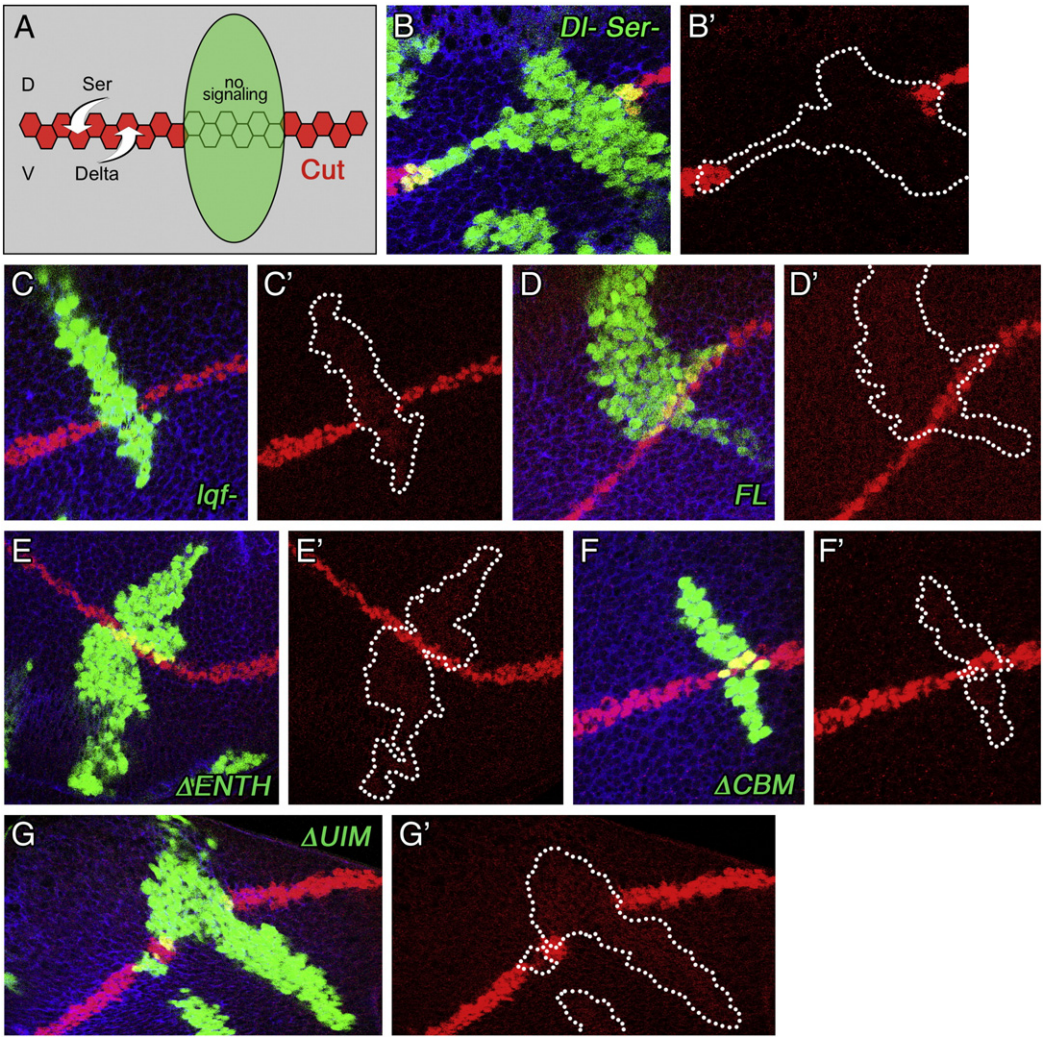

Fig. 4. Notch signaling in the wing disc requires Epsin's UIMs but none of the other Epsin module types individually. (A) A diagram of the D/V axis of the wing disc. Notch is activated in the red cells, monitored in these experiments by Cut expression. Serrate (Ser) activates Notch on the ventral side, and Delta activates Notch on the dorsal side of the midline. The green circle is a MARCM clone of homozygous mutant cells that express GFP and that span the D/V axis. Cut is not expressed in the mutant clone because the gene in question is required either for signal sending or signal receiving. (B–G′) Confocal microscope images of wing discs with MARCM clones. Dorsal is at top, and ventral is at bottom. In all panels, the homozygous mutant cells express GFP (green nuclei) and Cut-positive nuclei are red. Yellow cells express both GFP and Cut. Blue is f-actin. The rightmost of the image pairs shows Cut expression only, with the clone outlined. (B,B′) A *Dl*$^{-}$ *Ser*$^{-}$ clone generated in the genotype *hs-flp tub-gal4 UAS-ngfp/+*; *FRT82B Dl*^{rev} *Ser*^{RX82}*/FRT82B tub-gal80*. Notch is activated within the clone (yellow nuclei) at the edges by adjacent *Dl+ Ser+* cells. (C,C′) *lqf*$^{-}$ clone in the genotype *hs-flp tub-gal4 UAS-ngfp/+*; *lqf*$^{k.71}$ *FRT80B/tub-gal80 FRT80B*. (D–G′) Cells in the clones expressed Epsin only from the transgene indicated (see Fig. 2). The genotypes were *hs-flp tub-gal4 UAS-ngfp/+*; *Transgene/+ lqf*$^{k.71}$ *FRT80B/ tub-gal80 FRT80B*.

eye and wing imaginal discs indicates a specific failure of Notch signaling (Overstreet et al., 2004; Wang and Struhl, 2004, 2005). Second, Notch signaling is essential during *Drosophila* embryonic development, and consequently homozygous null mutants in core Notch pathway genes die during embryogenesis (Fleming et al., 1990; Lehmann et al., 1983; Nusslein-Volhard et al., 1984; Poulson, 1937). Likewise, mice with knock-out mutations in both Epsin genes (*epsin 1* and *epsin 2*) show typical Notch signaling defects throughout development, including embryogenesis (Chen et al., 2009). Finally, different mutant alleles of *lqf* affect viability and Notch-like morphology to a similar degree (Cadavid et al., 2000; Overstreet et al., 2003). Similarly, we always observed a correspondence between the ability of a transgene to rescue the lethality of *lqf* null mutants and Notch signaling defects later in development in either *lqf* null mutants or *lqf* hypomorphs (Fig. 2B and see later discussion).

Although the general results were similar in all assays, there were two subtle differences in the results in the wing as compared with the eye and early development assays. First, while *ΔUIM* retains some residual activity that rescues embryonic lethality and eye morphology slightly, it has no apparent activity in the Cut expression assay. Second, while *ΔENTH* rescues the lethality and eye morphology defects in *lqf* null mutants not quite completely, *ΔENTH*-expressing ligand cells activated Cut apparently normally in the wing. Most likely these discrepancies reflect differences in the sensitivities of the different assays in terms of how much Epsin activity is required to activate Notch, and/or in terms of how much Notch activation is required to observe a wild-type signal output. In addition, the levels of expression of the Epsin variants were tested in eye discs. It is possible that for some transgenes, wing disc expression levels may not correlate with the expression levels in eye discs (see Discussion).

Taken together, the results of these experiments indicate that Epsin's UIMs are vital to Epsin's ability to enable Delta or Serrate signaling. In contrast, the ENTH domain is required only for maximal Epsin activity, and in an otherwise intact Epsin protein, the CBMs, the DPW motifs, and the NPF motifs are each unnecessary.

Either UIM is sufficient for Epsin function in ligand signaling

Epsin has two UIMs (Fig. 6A) and we were curious to know whether or not both of Epsin's UIMs are required for ligand cell signaling. To test

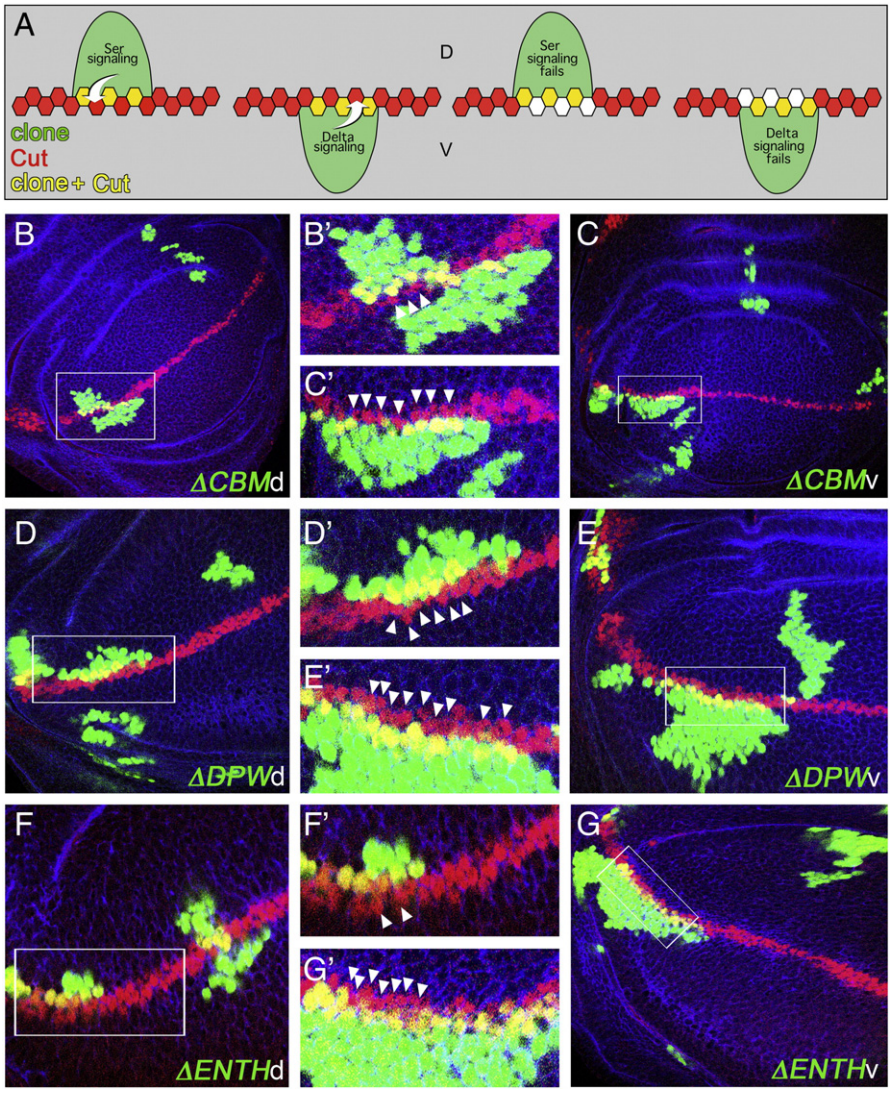

Fig. 5. Delta and Serrate signaling each require Epsin's UIMs but none of the other Epsin module types individually. (A) A diagram of clones at the dorsal/ventral boundary in the wing disc is shown. Serrate (Ser) signaling from the dorsal (D) side activates Cut expression in ventral midline cells and Delta signaling from the ventral side (V) activates Cut expression in dorsal midline cells. Failure of Ser signaling by cells within a dorsal mutant clone is detected by the absence of Cut in ventral midline cells and failure of Delta signaling by cells within a mutant clone is detected by the absence of Cut in dorsal midline cells. (B–G) Confocal microscope images of wing discs with MARCM clones (abutting the dorsal (d) or ventral (v) boundary) of cells that express Epsin only from the transgene indicated (see Fig. 2). The genotypes were *hs-flp tub-gal4 UAS-ngfp/+; Transgene/+; lqf^{fL71} FRT80B/tub-gal80 FRT80B*. In all panels, *lqf*-homozygous cells express GFP (green nuclei) and Cut-expressing nuclei are red. Yellow nuclei express both GFP and Cut. Blue is f-actin. (B′–F′) Enlargements of the boxed areas in B–G are shown. White arrows point to Cut-expressing nuclei that most clearly indicate signaling from cells within the clone.

this, we first generated a *lqf-gfp* transgene in the context of the 16 kb genomic DNA used earlier with a deletion of UIM2 (*ΔUIM2*). In transformed flies, *ΔUIM2* rescued the lethality of *lqf*$^{-}$ null mutants and the viable flies were almost completely wild-type morphologically (Fig. 6B,C). Likewise, in *ΔUIM2*-expressing *lqf*$^{-}$ null clones in the wing disc, Notch is activated by both Delta and Serrate (Fig. 6D). We conclude from these results that only a single UIM is essential. To test if the single UIM could be UIM2 instead of UIM1, we first generated a transgene identical to *ΔUIM2*, except that three UIM1 consensus Glutamic acid codons were changed to Alanine codons (*UIM1$^{EEE/AAA}$ΔUIM2*; Fig. 6B). Mutation of the N-terminal acidic UIM residues has been shown to eliminate the ability of UIM peptides to bind Ubiquitin *in vitro* (Fisher et al., 2003). *UIM1$^{EEE/AAA}$ΔUIM2* had only a very small amount of Epsin activity in the *lqf*$^{-}$ phenotypic rescue assays (Fig. 6B), and likewise in *lqf*$^{-}$ wing disc clones spanning the D/V boundary, *UIM1$^{EEE/AAA}$ΔUIM2* did not promote Cut activation (Fig. 6D). Thus, the UIM1 mutations rendered *UIM1$^{EEE/AAA}$ΔUIM2* essentially inactive. Next, we added back to *UIM1$^{EEE/AAA}$ΔUIM2* the UIM2 sequences to generate *UIM1$^{EEE/AAA}$* (Fig. 6B). We found that *UIM1$^{EEE/AAA}$* had significant activity, just below that of *ΔUIM2* (Fig. 6B). *UIM1$^{EEE/AAA}$* rescued *lqf* null mutants to nearly wild-type (Fig. 6B,C) and in about half the dorsal or ventral *lqf*− wing disc clones assayed, *UIM1$^{EEE/AAA}$* restored the ability of Serrate or Delta, respectively, to signal (Fig. 6D). We conclude that only one UIM is necessary for very nearly full Epsin activity in ligand cells, and that either UIM1 or UIM2 is sufficient.

The CBMs, DPWs and NPFs are redundant with each other

Earlier we discovered that Epsin's function in Notch ligand cells is independent of its CBMs, DPW motifs, or NPF motifs in an otherwise intact protein. To determine whether the C-terminal modules are required at all, we generated a *lqf-gfp* transgene containing only the ENTH domain and the UIMs (*ENTH-UIM*). *ENTH-UIM* has no ability to rescue the mutant phenotypes of *lqf*$^{-}$ mutants (Fig. 7A,C). As we have shown that each of the C-terminal module types is dispensable, the C-terminal module types must be redundant for Epsin's role in ligand signaling. To explore the nature of the redundancy, we generated two additional *lqf-gfp* transgenes, one with deletions of the CBMs

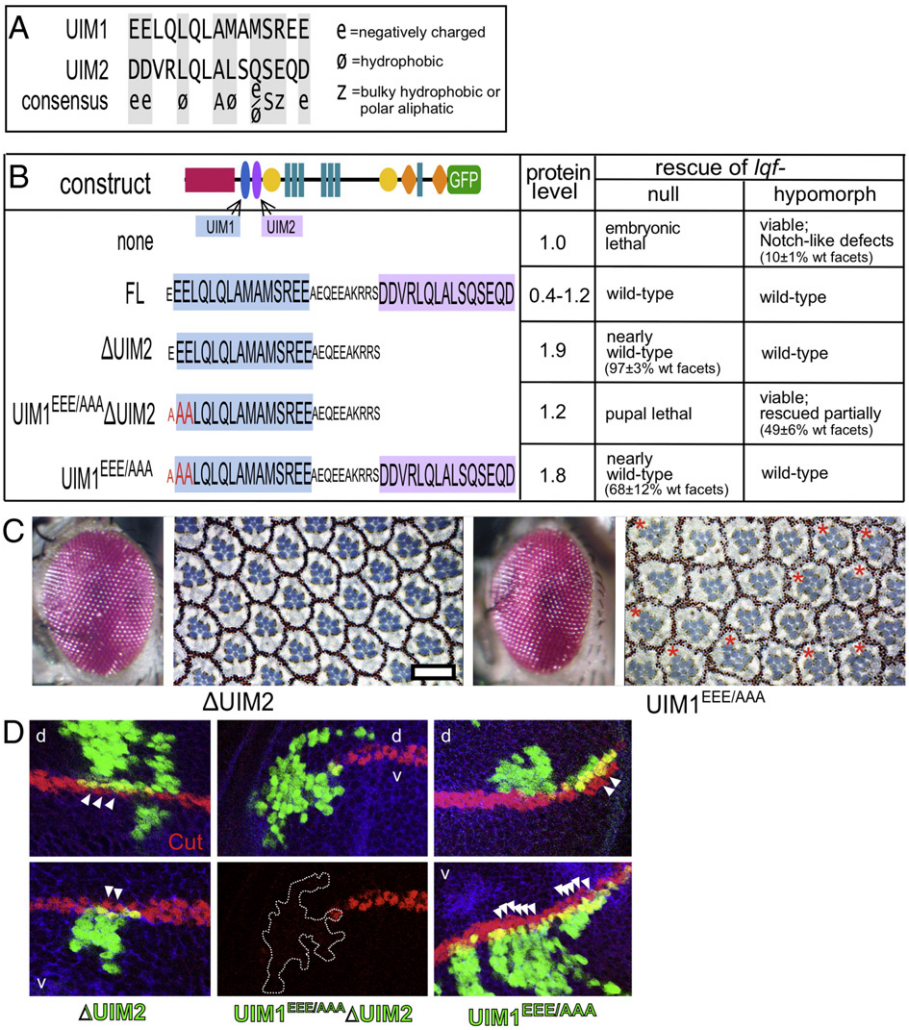

Fig. 6. Epsin with only one UIM supports both Delta and Serrate signaling. (A) UIM1 and UIM2 of *Drosophila* Epsin are shown and each conforms to the consensus for single-sided UIMs (Hirano et al., 2006). (B) At left, alterations in the UIM region of the three different Epsin variants indicated are shown. The transgenes in this experiment were all introduced into the same genomic location using a PhiC31 integrase vector (Materials and methods). The red amino acids are altered. See Fig. 2 legend for explanation of the remainder of the table. (C) Eyes of *lqf* null (lqf^{L71}/lqf^{ARI}) adults expressing the Epsin variant indicated from a single copy of a transgene (external eyes at left and tangential sections at right) are shown. The red asterisks indicate mutant facets with extra photoreceptor cells. (D) Confocal microscope images of wing discs with MARCM clones of *lqf* null (lqf^{L71}) cells (green nuclei) expressing the Epsin variant indicated later. Yellow nuclei express both GFP and Cut. Blue is f-actin. The *ΔUIM2* and $UIM^{EEE/AAA}$ clones shown abut the dorsal (d) or ventral (v) border of the D/V axis (see Fig. 5 and legend), and the $UIM^{EEE/AAA}$ ΔUIM2 clones span the D/V axis (see Fig. 4 and legend). The central bottom image shows only Cut expression with the clone outlined. White arrows point to Cut+ nuclei (near the center of the clone) that indicate most clearly that the cells within the clone are signaling. The genotypes were *hs-flp tub-gal4 UAS-ngfp/+; Transgene/+; lqf^{L71} FRT80B/tub-gal80 FRT80B.*

and one DPW cluster (*ΔCBM−ΔDPW1*), and the other with the CBMs and both DPW clusters deleted (*ΔCBM−ΔDPW*). *ΔCBM−ΔDPW1* rescues *lqf* null mutants to wild-type, and *ΔCBM−ΔDPW* has just slightly less activity in that the null eye has a small number of mutant facets (Fig. 7A,B). Similarly, in the wing, each transgene restores the ability of Serrate and Delta to signal (Fig. 7C). This result indicates that Epsin functions nearly normally in ligand cells with only one of the C-terminal module types intact — the NPFs.

We wondered whether like the NPFs, the DPW motifs or the CBMs would support Epsin function independent of the other two module types. Instead of testing this with additional deletion variants, we attempted to generate "minimal Epsin-GFP" genes in the context of the 16 kb *lqf* genomic DNA fragment, containing only the ENTH domain, UIM1, and four copies of either the CBMs (*4XCBM*), DPW clusters (*4XDPW*), or NPF motifs (*4XNPF*). We took this approach in order to address whether or not there are unknown motifs in the C-terminus that may be contributing to Epsin function. We detected no Epsin-GFP protein in flies transformed with *4xCBM* or *4XDPW* (data not shown). In contrast, the 4XNPF protein was expressed at high levels and the transgene rescued *lqf*⁻ mutant phenotypes significantly (about as well as *ΔENTH* does) (Fig. 7A,B). However, *4XNPF*-expressing clones abutting the D/V wing boundary did not activate Cut detectably (Fig. 7C). This was surprising as ΔENTH activity is similar to 4XNPF in the eye, and ΔENTH supports Cut activation (Fig. 5E). A likely possibility is that the transgene is expressed at somewhat lower levels in the wing than in the eye or in early development, and that this expression level is beneath the threshold for detectable Cut expression. Alternatively, lower levels of NPF binding proteins in the wing than in the eye could account for the difference in *4XNPF* activity (see Discussion).

We conclude that there is functional redundancy among the C-terminal modules, and that the NPF motifs alone were sufficient to provide a significant portion of the function of this region. Additional experiments are needed to determine whether the CBMs or DPW motifs alone would provide as much Epsin function in ligand cells as the NPFs do.

Deletion of UIMs, CBMs, DPWs or NPFs interferes with Epsin binding in vitro to Ubiquitin, Clathrin, α-Adaptin, and Eps15, respectively

Protein–protein interactions between Epsin's various modules (UIMs, CBMs, DPWs, and NPFs) have been characterized previously in

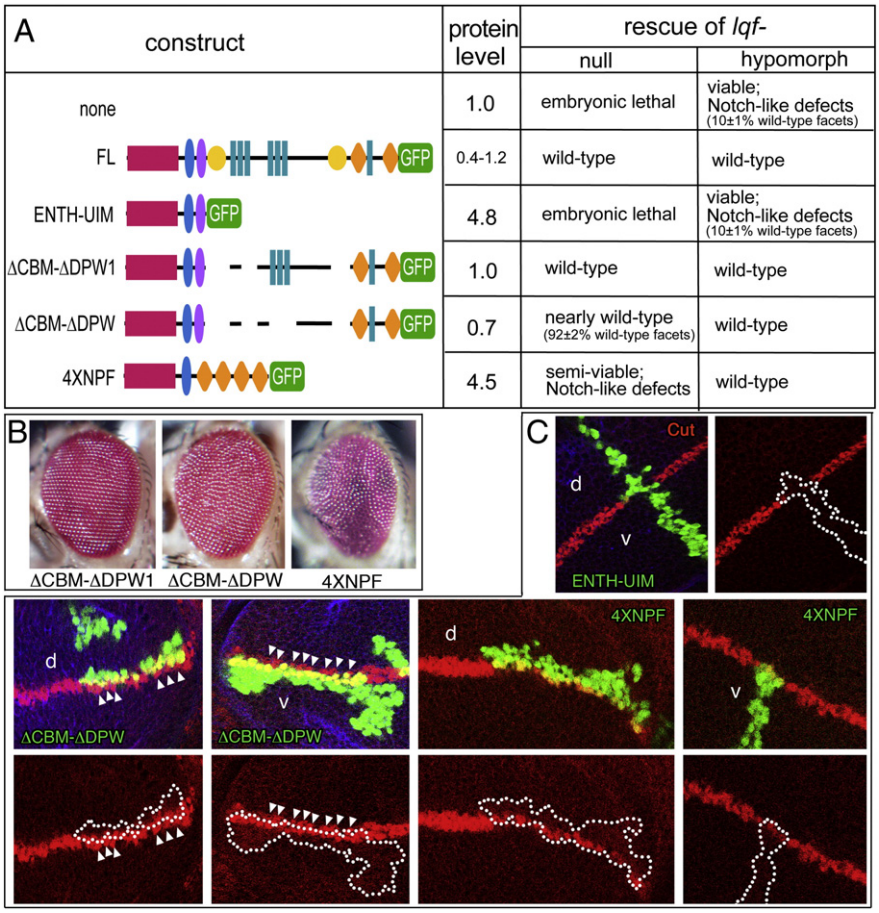

Fig. 7. Epsin's C-terminal modules are redundant with each other. (A) At left are diagrams of full-length (FL) Epsin-GFP protein and three deletion derivatives (described in text) expressed by P element transgenes. *4xNPF* was introduced using a PhiC31 integrase vector (Materials and methods). See Fig. 2 legend for explanation of the remainder of the table. (B) Shown are eyes of rescued $lqf^{\Delta RI}/lqf^{L71}$ adults expressing the Epsin variant indicated from a single transgene copy. (C) Confocal microscope images of wing discs containing lqf^{L71} homozygous MARCM clones that also express the Epsin-GFP variant indicated (green). Cut expression (red) indicates Notch activation at the D/V boundary. Yellow cells express both Epsin-GFP and Cut. Blue is f-actin. The images are in pairs, where one image shows Cut expression only and the clone is outlined. The clone expressing ENTH-UIM spans the D/V boundary and all of the other clones abut the boundary from either the dorsal (d) or ventral (v) side. White arrows mark the Cut + nuclei that most clearly indicate that cells within the clone are signaling.

a variety of different contexts (see Introduction). Nevertheless, we wanted to test whether the deletions of modules were indeed disrupting the ability of the particular *Drosophila* Epsin variants we generated to interact with the expected *Drosophila* proteins. To this end, we generated GST fusion proteins in bacteria with Ubiquitin (GST-Ub), the Clathrin heavy chain "terminal domain" (GST-ChcTD), the EH domain (NPF-binding region) of Eps15 (GST-Eps15EH), and the "ear domain" of α-Adaptin, the AP-2 subunit that binds Epsin (GST-α-AdaEar). Purified GST fusion proteins were tested in GST "pull-down" experiments for interaction with wild-type *Drosophila* Epsin (FL) or Epsin deletion derivatives, all without GFP tags, purified in bacteria as MBP fusions. As a control for artifactual results, Epsin and each derivative were also tested under similar conditions for binding to GST alone and none of the proteins bound GST (data not shown).

First, we found that under conditions in which FL bound to GST-Ub, ΔUIM2 (Fig. 2) bound to GST-Ub also, but ΔUIM (Fig. 2) lost the ability to interact with GST-Ub (Fig. 8A). We conclude that the UIMs are required not only for Epsin function but also for Epsin to bind Ub *in vitro*. In addition, there is a correlation between Epsin's ability to bind Ub *in vitro* and its ability to function *in vivo*; UIM1, which is sufficient for Epsin function without UIM2 in an otherwise intact protein, is also sufficient for Ub binding *in vitro*.

Next, we found that GST-ChcTD pulled down FL, but pulled down neither ΔCBM nor ΔCBM-ΔDPW (Figs. 2, 8B). Thus, deletion of the CBMs hampered the ability of Epsin to bind Clathrin *in vitro*. Similarly, we found that deletion of the NPF motifs (ΔNPF, Fig. 2) prevented Epsin from interacting *in vitro* with the EH domain of Eps15 (Fig. 8C), and deletion of the two clusters of DPW motifs (ΔDPW, Fig. 2) weakened the *in vitro* interaction between α-AdaEar and Epsin (Fig. 8D). We conclude that at least *in vitro*, deletion of the characterized protein binding motifs is indeed interfering with the ability of Epsin to bind the proteins predicted.

The lipid-binding function of the ENTH domain, but not the Cdc42 GAP interaction function, is required for Epsin's role in ligand cells

We found earlier that the ENTH domain, while not essential for Epsin function in ligand cells, is required for maximal Epsin activity (Fig. 2). Two functions have been proposed for the ENTH domain. First, the ENTH domain brings Epsin to the plasma membrane through interactions with PIP_2 (Aguilar et al., 2003; Itoh et al., 2001). Plasma membrane binding of the ENTH domain promotes endocytosis in two ways: it brings the other Epsin modules to the membrane and also induces membrane curvature (Ford et al., 2002). Second, the ENTH domain binds Cdc42 GAP and thus regulates actin dynamics and cell polarity (Aguilar et al., 2006a). The observation that ΔENTH protein localizes to the plasma membrane less well than wild-type Epsin (Fig. S3) is consistent with either function, as ENTH domain/Cdc42 GAP interactions are expected to occur at the plasma membrane (Aguilar et al., 2006a). Expression in yeast of either full-length *Drosophila* Epsin or the ENTH domain only rescues the lethality of yeast mutants lacking Epsin (*ent1Δent2Δ*) (Overstreet et al., 2003). As the lethality of yeast

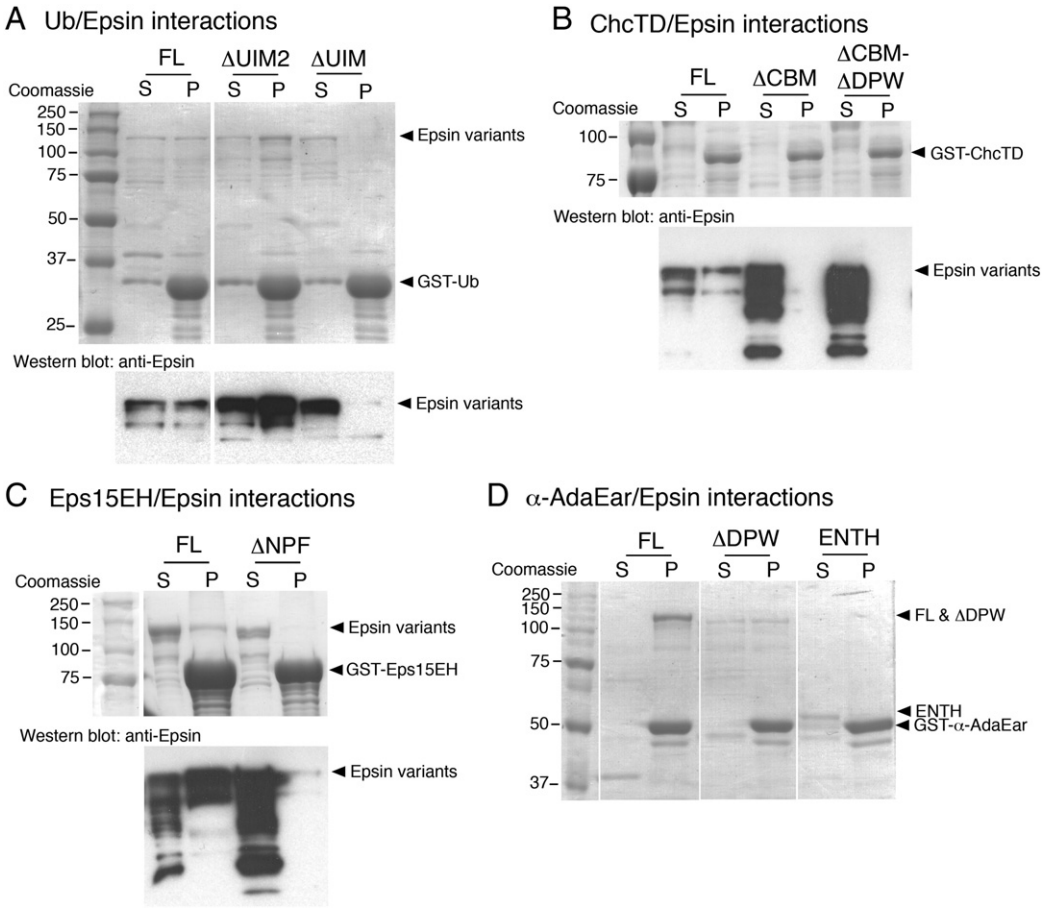

Fig. 8. GST pull-down assays with Epsin derivatives and Epsin-binding proteins. Coomassie-stained protein gels and protein blots of the same gel hybridized with anti-Epsin are shown. (In D, only a Coomassie-stained gel is shown.) At left are size markers (units are kD). The GST fusion proteins indicated were immobilized on columns and their ability to bind the MBP-Epsin variants indicated (described in text) was tested. S = supernatant (unbound fraction), P = pellet (bound fraction). Equivalent amounts of each Epsin variant were used in each assay. See Materials and methods for complete experimental details. (A) An assay of GST-Ub binding to each of the MBP-Epsins indicated is shown. Each S lane represents 1/80 of the unbound fraction, and each P lane is 1/3 of the bound fraction. The panels below show only the top portion of the blot. GST-Ub = 36 kD. Sizes of MBP fusion proteins: FL (Lqf2) = 118 kD; ΔUIM2 = 116 kD; ΔUIM = 113 kD. (B) An assay of GST-ChcTD binding to each of the MBP-Epsins indicated is shown. Each S lane represents 1/24 of the unbound fraction, and each P lane represents 2/5 of the bound fraction. The Epsin variants were not visible with Coomassie staining, but were detectable on the blot probed with anti-Epsin. GST-ChcTD is 92 kD. Sizes of MBP fusion proteins: ΔCBM = 117 kD; ΔCBM − ΔDPW = 110 kD. (C) An assay of GST-Eps15EH binding to each of the Epsins indicated is shown. Each S lane represents 1/24 of the unbound fraction, and each P lane represents 2/5 of the bound fraction. GST-Eps15EH is 84 kD, MBP − ΔNPF = 111 kD. (D) An assay of GST-α-AdaEar binding to each of the MBP-Epsins indicated is shown. Each S lane represents 1/80 of the unbound fraction, and each P lane represents 1/4 of the bound fraction. GST-α-AdaEar is 56 kD. Sizes of MBP fusion proteins: ΔDPW = 112 kD; ENTH = 73 kD.

Epsin mutants is due to the loss of the ENTH domain's ability to polarize actin via Cdc42 regulation (Aguilar et al., 2006a), we reason that the ENTH domains of *Drosophila* and yeast Epsin likely interact with Cdc42 GAP the same way. Distinct ENTH domain amino acids have been identified that are specific either for plasma membrane or Cdc42 GAP binding (Aguilar et al., 2006; Itoh et al., 2001). To determine which of the two functions of the ENTH domain is required for maximal Epsin activity in ligand cells, we generated *lqf-gfp* transgenes that generate Epsins with mutations that alter amino acids key to one function or the other.

We first generated $ENTH^{T/D}$ and $ENTH^{FTVF/RDAA}$ transgenes which produce Epsin variants with alterations in amino acids required for Cdc42 GAP interaction. Each of them functions as well as the *FL* (normal Epsin) transgene (Fig. 9A,B). Thus, we conclude that the actin organizing function is not essential to Epsin's role in Notch ligand cells. By default, the ENTH domain's role in PIP_2 binding is implicated as its function in Notch ligand cells. We tested this directly by generating transgenes that express Epsin variants with alterations in amino acids needed for PIP_2 binding. The transgene $ENTH^{RWRK/AAAA}$ produces an Epsin with four key amino acid changes (Fig. 9A). $ENTH^{RWRK/AAAA}$ provides only a barely detectable amount of Epsin activity, but the protein accumulates only to a small fraction of wild-type Epsin levels (Fig. 9A). Because the low levels of $ENTH^{RWRK/AAAA}$ protein make these results difficult to interpret, we generated $ENTH^{R/A}$, which contains a mutation that changes only one key residue to Alanine (Fig. 9A). $ENTH^{R/A}$ protein accumulates to normal levels and although it retains more activity than ΔENTH (see Fig. 1B), its activity is noticeably below wild-type (Fig. 9A,B). As $ENTH^{R/A}$ protein differs from wild-type Epsin (FL) by only one amino acid and yet has less activity than wild-type Epsin, this result supports the idea that the ENTH domain is required for its PIP_2 binding function.

Discussion

Epsin is a complex multi-modular protein that functions differently in different contexts. In *C. elegans*, *Drosophila*, and mice, Epsin is needed specifically in Notch ligand cells (Chen et al., 2009; Overstreet et al., 2004; Tian et al., 2004; Wang and Struhl, 2004). The structure/function analysis of Epsin we performed here shows that modules of Epsin associated with the internalization step of endocytosis – the lipid binding function of the ENTH domain and the C-terminal modules that bind proteins present in Clathrin-coated vesicles – are required for Epsin's function in Notch ligand cells. In addition, we have shown that a UIM

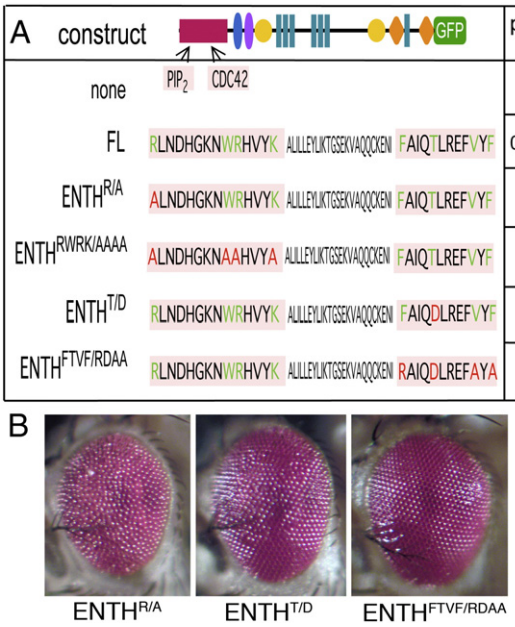

Fig. 9. The lipid binding function of the ENTH domain affects Epsin's function in Notch signaling. (A) At left, alterations of the ENTH domain in three different Epsin variants indicated are shown. The pink shaded box at the left contains amino acids (green) important for plasma membrane lipid (PIP_2) binding, and the pink box to the right contains amino acids important for binding Cdc42-GAP. The amino acids in red are altered. The transgenes in this experiment were all introduced into the same genomic location using a PhiC31 integrase vector (Materials and methods). See Fig. 2 legend for explanation of the remainder of the table. (B) Eyes of *lqf*− (lqf^{ARI}/lqf^{L71}) adults expressing the Epsin variant indicated from a single transgene copy.

is necessary. We discuss later how these results lead to a specific model for Epsin function in ligand cells.

The actin organizing function of the ENTH domain is not essential for Epsin function in Notch ligand cells

The dispensability of the Cdc42 GAP binding function of the ENTH domain suggests that in ligand cells the primary role of *Drosophila* Epsin, unlike yeast Ent1, is not regulation of actin dynamics. The other known function of the ENTH domain is the endocytic function, and the results suggest that the ability of the ENTH domain to interact with PIP_2 explains why it is needed for maximal Epsin function in Notch ligand cells. These observations are consistent with the lack of typical Notch signaling defects in *Drosophila cdc42* mutants (Genova et al., 2000). In contrast, flies with mutations in genes for either of two actin regulators, the Arp2/3 complex and WASp, do have notal bristle defects indicative of Notch signaling failure (Rajan et al., 2009). The results we present here are not in conflict with the findings of Rajan et al. (2009). The notal bristle phenotype described is not due to failure of the Epsin-dependent endocytosis of ligand that activates Notch in all cell types, but instead to failure of ligand transcytosis required in only some cell types to relocalize ligand prior to signaling (see Weinmaster and Fischer, 2011). The absence of the Arp2/3 complex or WASp in mutants inhibits signaling by blocking traffic of endocytosed Delta to apical microvilli of sensory organ precursors (Rajan et al., 2009). Whether or not Delta transcytosis in sensory organ precursors also depends on Epsin is unknown. If Epsin is involved, it may be interesting to use the Epsin variant transgenes we generated to determine whether or not the Cdc42 GAP interaction function of the ENTH domain is required.

A UIM is critical to Epsin activity in Notch ligand cells, and a single UIM is sufficient

There are two types of UIMs: single-sided UIMs that bind one Ubiquitin, and double-sided UIMs that bind two Ubiquitins simultaneously (Hirano et al., 2006). As the affinity between a UIM and Ubiquitin is low, successful interaction between a mono-ubiquitinated protein and a UIM-containing protein is thought to require either one double-sided UIM, or two single-sided UIMs (Barriere et al., 2006; Hawryluk et al., 2006; Hirano et al., 2006; Madshus, 2006). Epsins have single-sided UIMs, and so the observation that only one single-sided UIM is required for *Drosophila* Epsin function in Notch signaling is unexpected. The simplest explanation is that Notch ligands use multiple mono-Ubiquitins or Ubiquitin chains as a signal for Epsin-mediated internalization (Heuss et al., 2008; Traub and Lukacs, 2007). Two distinct Lysine residues in the intracellular domains of both Delta and Serrate have been

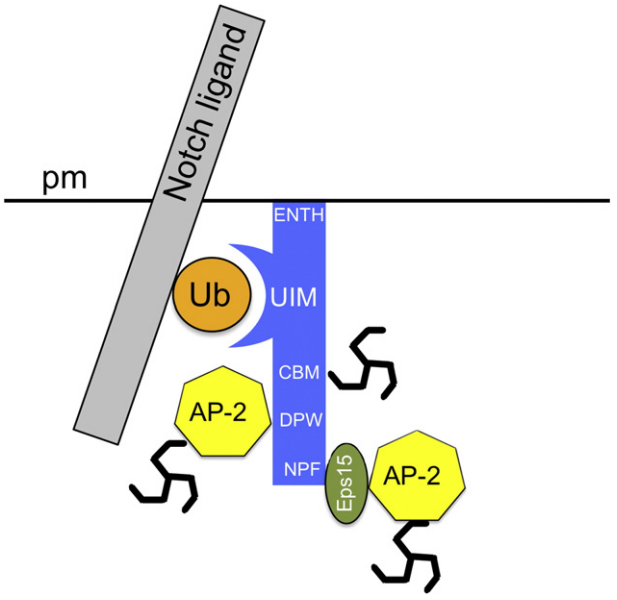

Fig. 10. A model for Epsin function in Notch ligand cells. We propose that in its function in Notch ligand cells, Epsin serves as the cargo selection subunit of a Clathrin adapter. Through its UIMs, Epsin binds ubiquitinated Notch ligands at the plasma membrane. The ENTH domain aids in Epsin localization to the plasma membrane. The Epsin C-terminus binds Clathrin both directly and indirectly to promote Clathrin-dependent ligand endocytosis. Epsin cooperates with another Clathrin-binding protein, likely AP-2, and also with an EH-domain protein, likely Eps15.

implicated as important for the function of each ligand. In the case of Serrate, simultaneous mutation of both of these Lysines results in a Serrate ligand that can neither activate Notch nor be endocytosed in wing discs (Glittenberg et al., 2006). These observations identify two particular Lysines as candidates for the critical Ub attachments, but do not distinguish whether one or both Lysines are required. In the case of Delta, single mutation of either of two specific Lysines results in accumulation of Delta at the cell surface of eye discs and failure to signal (Parks et al., 2006). Although Delta is thought to be mono-ubiquitinated (Deblandre et al., 2001), these results suggest the possibility that Delta is multiply mono-ubiquitinated. An alternative explanation for Epsin's ability to promote ligand endocytosis with a single UIM is that mono-ubiquitinated ligands cluster to generate an environment where multiple Ubiquitins attract Epsin to ligand at the plasma membrane (Le Borgne and Schweisguth, 2003).

Epsin function in Notch ligand cells is not as a monomeric Clathrin adapter

There is compelling evidence that in somatic cells, Notch ligand endocytosis associated with signaling is Clathrin-dependent. First, there are exceedingly strong genetic interactions between the *Clathrin heavy chain* (*Chc*) gene and *lqf*, the gene for Epsin. Flies with only one *Chc+* gene copy are wild-type, but this condition is lethal in homozygotes for a normally viable hypomorphic allele of *lqf* (Cadavid et al., 2000). Second, the Clathrin-coated vesicle uncoating protein Auxilin is, like Epsin, required specifically for Notch signaling in *Drosophila* and in ligand cells (Eun et al., 2007, 2008; hagedorn et al., 2006; Kandachar et al., 2008). Given the clear involvement of Clathrin and the lack of strong genetic interaction between *α-Adaptin* (the gene for an AP-2 subunit) and *lqf* (Cadavid et al., 2000), the simplest model for Epsin function in Notch signaling was as an adapter protein that links Clathrin and the plasma membrane, independent of AP-2. This model predicted that direct interaction between Epsin and Clathrin would be necessary, and thus the most surprising result of this work is that deletion of the CBMs had no detectable effect on Epsin activity. The dispensability of the CBMs rules out models where Epsin acts as a monomeric Clathrin adapter that links ligand to Clathrin cages.

In the *Drosophila* female germline, Notch signaling requires Epsin but neither Clathrin nor Auxilin (Banks et al., 2011; Windler and Bilder, 2010). Although this is surprising, Epsin has been shown to function in Clathrin-independent internalization of ubiquitinated transmembrane cargos in vertebrate cell culture (Sigismund et al., 2005). Epsin must therefore function differently in Notch signaling in the female germline than in somatic cells. We speculate that the ENTH domain and UIMs may be required in germline cells to guide the ubiquitinated proteins into an endocytic vesicle (see Aguilar and Wendland, 2006). However, it is not clear how any of the characterized modules within Epsin's C-terminus might be involved in Clathrin-independent endocytosis (Mayor and Pagano, 2007). It would be of interest to use the transgenes we have generated to determine which motifs are required in the female germline. Additional experiments could potentially identify unknown C-terminal interaction motifs used in Clathrin-independent endocytosis.

Does Epsin function in the same way in the embryo, eye, and wing?

We began the experiments with the assumption that Epsin functions through the same mechanism in all signaling contexts, and thus we expected the same Epsin modules would be required for Epsin function in all contexts. Epsin appears to be required in every Notch signaling event and thus could be regarded as a core component of Notch signaling. It therefore seems reasonable to expect that Epsin would function in the same manner in all tissues. As described earlier, the female germline is apparently an exception. Nevertheless, in the three assays we used for Epsin activity – rescue of lethality and eye morphology defects due to *lqf* mutations and rescue of the ability of *lqf* null cells to activate Cut expression in cells at the D/V boundary in the wing disc – we detected only subtle differences between the eye and the wing in the activity of two Epsin variants, ΔENTH and ΔUIM. (The only major difference was with the highly artificial Epsin variant, 4XNPF.) Despite these differences, we think that Epsin likely functions the same way in the eye and wing, as well as during embryogenesis. For one, the differences in activity we observed may be explained easily without invoking different mechanisms for Epsin in the eye and wing (see Results). Importantly, we never observed even one case where modules were essential in one context (embryogenesis, eye, or wing development) and dispensable in another one. In fact, it is possible to observe all-or-none differences in requirements for Epsin modules. We have discovered that Epsin has a function outside of Notch ligand cells and we did find modules that are dispensable completely in this context yet absolutely essential for Epsin's function in ligand cells (B.C., X.X., and J.A.F., manuscript in preparation).

A model for Epsin function in Notch ligand cells

Notch ligands require ubiquitination and (usually) Clathrin-dependent endocytosis, and formation of Clathrin-coated vesicles requires adapter proteins that link the plasma membrane with Clathrin (Owen et al., 2004). The absolute necessity of at least one UIM and the observation that the lipid-binding function of the ENTH domain plays a role in ligand cells suggests that Epsin indeed binds ubiquitinated Notch ligands at the plasma membrane. However, as an Epsin derivative lacking CBMs functions as well as wild-type Epsin in ligand cells, the essential role of Epsin in Notch signaling cannot be as a monomeric Clathrin adapter that links Clathrin directly to ligand at the plasma membrane. As any pair of the three types of modules is sufficient for Epsin function (CBMs + DPWs, CBMs + NPFs, or DPWs + NPFs), Epsin must be able to support Notch activation by linking ligand to Clathrin in a variety of different ways (Fig. 10). We speculate that Eps15 is involved because of the three EH-domain proteins in *Drosophila* (Eps15, Dap160, Past1), none have Clathrin binding motifs, and Eps15 is the only one with motifs for a known Clathrin-binding protein (AP-2) (Koh et al., 2007; Olswang-Kutz et al., 2008). From analysis of mutant phenotypes and genetic interaction studies, there is no evidence for the involvement of Eps15 nor AP-2 in Notch signaling (Gonzalez-Gaitan and Jackle, 1997; Koh et al., 2007; Windler and Bilder, 2010). The results presented here suggest that Eps15 and AP-2 may play redundant roles in the presence of intact Epsin and this idea could be tested with additional genetic experiments. In light of the evidence indicating a requirement for Clathrin in ligand cells (outside of the germline), the results suggest that Epsin is required absolutely for Notch signaling not because it generates a special endocytic environment, but simply because it is the only UIM-containing endocytic protein with the appropriate complement of interaction modules to target ubiquitinated cargo to Clathrin-coated vesicles.

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